AD NUMBER

AD414906

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Foreign Government Information; MAY 1963. Other requests shall be referred to US Army Biological Laboratories, Fort Detrick, MD 21701.

AUTHORITY

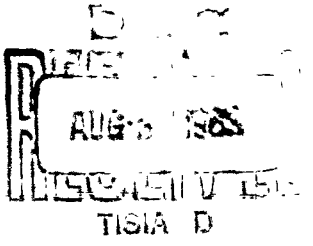
SMUFD, D/A ltr, 4 Feb 1972

FAST LABORATORY DIAGNOSIS BY MEANS OF FLUORESCENT ANTIBODIES

TRANSLATION NO.

786

MAY 1963



U.S. ARMY BIOLOGICAL LABORATORIES
FORT DETRICK, FREDERICK, MARYLAND

FAST LABORATORY DIAGNOSIS BY MEANS OF FLUORESCENT ANTIBODIES

- Yugoslavia -

[Following is a translation of an article by Miro Gerbec, docent dr., Lt. Col. of medical services (Sanitetski potpukovnik), of the Microbiological Institute, Military Medical Academy in Belgrade, published in the Croat-language periodical Vojnosanitetski Pregled (Army Medical Review), Vol. XVIII, No.6/7, Belgrade, 1961, pages 574-579.]

All branches of microbiology (bacteriology, virology, parasitology, and mycology) have made great strides forward in the last decade. We have completed our knowledge about metabolism of microorganisms, about factors of the growth and breathing of microorganisms. On the basis of these deeper findings we prepare elective and selective bacteriological bases in order to isolate pathogenic microorganisms more easily. In virology, we have completed the technique of texture cultures, which made it possible to discover more than one hundred new human viruses. We have enriched our knowledge in the field of immunology, we have worked out reliable tests for immunological identification of various microorganisms. We discovered and continue to discover all the time new and very effective antibiotics, which have become irreplaceable in the treatment of many contagious diseases, especially those caused by bacteria. However, we cannot always take advantage of the existence of effective antibiotics, because a precise microbiological diagnosis usually is made late. The inadequacy of microbiological laboratory methods with regard to speed has induced many authors to seek new ways and new solutions.

The search for new and fast microbiological methods encountered insurmountable difficulties, as long as the search was oriented to speed up the growth of microorganisms, to the shortening of individual laboratory procedures, etc., because it turned out that at present we are unable to influence basic processes of the life of microorganisms. Until recently, a reliable microbiological identification of some agents consisted in the isolation of microorganisms in a pure culture. A pure culture of some agent was the starting point for all further laboratory operations. However, serological reactions are unsuitable for an early

diagnosis. They represent merely a manifestation of immunological processes which can be observed in a laboratory, and which take place as a result of the conflict between a macroorganism and a microorganism. Consequently they cannot be used profitably for a retrogressive determination of some disease of an infectious nature. In order to get out of this blind alley, it was necessary to proceed in a new direction, and to go around the classical method of cultivation of microorganisms. Only in that way can we gain time. The battle for saving time in modern infectology is of a prejudicial significance, if we want to exploit the therapeutic action of antibiotics and hemotherapeutics, and if we want to influence substantially the course of the disease. Before the era of antibiotics and modern hemotherapeutic means, laboratory diagnosis was used as a guide for prognosis and for the final outcome of the disease, for scientific and epidemiological studies, but otherwise clinical doctors did not benefit from it directly. Today, when medicine has put into the hands of the physician powerful means for a radical intervention, by which the physician can influence substantially the course of the disease, an early diagnosis has become a categoric imperative.

In solving this problem, we followed a new revolutionary way. We have discovered a new method to make an early and fast microbiological diagnosis, a method of coloring by fluorescent antibodies. This method consists in the fact that prosthetic groups, which are easily visible, combine with the globulin of antibodies and do not affect its reactivity and specificity. We can use them to dye a fixed preparation of an antigen. The introduction of a prosthetic group (fluorescin) in a molecule of globulin of the antibody is called noting or marking ("obelezavanje" or "markiranje"). The process of coloring does not follow the chemical laws, according to which aniline dyes are combined with individual elements of a big preparation (texture, sponge, emulsion, culture, etc), but rather immunological laws. The antibody which has been marked previously with fluorescin combines with an antigen, and the "coloring" is the result of a specific reaction antigen-antibody, which can be observed in a fluorescent microscope.

History

As early as in 1934, Marrack (1) has shown that it is possible to combine colors with the molecules of antibodies without losing their specificity. Coons (2) was the first to succeed to mark antibodies with fluorescent colors on the basis of work carried out by Creech and Jones (3). He combined successfully beta-anthrylisocianate with an antiserum of pneumococci of type 3. The marked specific serum retained its capacity to combine with the complement, to agglutinate with pneumococci of type 3 (but not with other types), and passive sensitization for an anaphylactic shock. Homologous pneumococci, treated with marked antiserum, had a fluorescent light bluish color, when they were exposed to ultraviolet light.

The unfavorable aspect of blue fluorescence is that the texture also has the same fluorescent color, and as a result it is more difficult to find the pneumococci in the texture. For that reason Coons and his co-workers (4) used isocianate fluorescin, which has a greenish light. An antiserum marked with this fluorescent color made it possible to observe the antigen (microorganism) in the texture. Later on Coons and Kaplan (5, 6) perfected the technique of conjugation (combination of fluorescin with globulin) and reviewed the non-specific combination of fluorescent antibodies with the texture by the absorption of the antiserum with a texture extract.

Another improvement of the technique of coloring by fluorescent antibodies resulted from the findings of Riggs (7) that fluorescin isotiocianate has two favorable properties of isocianate combinations and that it is very stable. In addition, the preparation has the advantage that it does not require organic solvents for combinations with globulin molecules - such as acetone or dioxane-, which reduce the capacity of the antibodies (8), as noted previously.

Later on we produced synthetically other colors which produce red or yellow fluorescent light. Silverstein (9) used a tetraethyl derivative of fluorescin, namely rhodamine, which produces a red fluorescent light. Hiramoto and his co-workers (10) produced synthetically tetramethyl rhodamine, which produces yellow fluorescent light, and Chadwick and his co-workers (11) produced synthetically sulphanyl-chloride derivative lysamine of rhodamine RB200, which produces red-orange fluorescent light. Smith and his co-workers (12) found that is is possible to combine dyes with a normal serum, which is not absorbed by a texture powder to eliminate non-specific characteristics of the coloring, and that can be used very well for non-specific contrast coloring of texture. This combination used for non-specific coloring of the background of the preparation does not cover or prevent the coloring by means of specific marked antibodies. The contrast coloring facilitates the observation of the distribution of antigens, small bacteria or virus aggragates in textures and cell cultures. Another great advantage of the fluorescent light in various colors lies in the fact that the coloring can be done with two marked antiserums, which produce fluorescent light of different colors and make it possible to identify two different antigens at the same time.

General Observations

The above explanations show that the method of dyeing by means of fluorescent antibodies represents a very significant supplement of standard laboratory methods for the detection and identification of microorganisms as well as for the studies of their antigenic similarities. This method makes it possible to discover even such small numbers of microorganisms which are not discovered by other standard methods. For that

reason the method of dyeing by means of fluorescent antibodies is especially beneficial for epidemiological studies, because in such studies a fast diagnosis is of prejudicial importance. The vectors of contagious diseases frequently carry agents in an exceptionally small number, but even these can be discovered by this method. In case we apply biological agents in war, this method is the best, because it is the fastest and the most reliable one.

Other good features of this method:

The agent can be discovered even when it is not alive, which is especially important with regard to malignant infections.

The agent or antigen can be discovered when mixed with many other agents or antigens, which makes it possible to study very mixed samples (stool, soil).

Self-agglutinable germs or non-agglutinable germs can be identified easily (because of the capsule-shaped wrapping).

The fluorescent marking of the antibodies can be used as some sort of "investigators" as investigators tracing minerals). They are very convenient for tracing alien antigens (bacteria, viruses, germs) in vivo. They can be used to locate antigens in an organism.

Fluorescent antibodies were applied beneficially also in those areas of medicine which up to now were subject to academic and speculative studies. Thanks to fluorescent antibodies, the pathogenesis of allergic ailments has become the object of exact laboratory research. Marshall (13) began in 1951 to study adrenocorticoafferent hormones by this method, and in this way he opened new facilities for hysto-chemistry, immuno-chemistry, and enzymology. This method was applied profitably in studies of the place and mechanism of the production of antibodies. Today, we are able to check and consider objectively many immuno-biological manifestations by means of fluorescent antibodies.

The method of fluorescent antibodies makes it possible to prepare specific fluoro-dyes for an unlimited number of antigens. It promises that very soon we shall have valuable laboratory means to identify promptly pathogenic and nonpathogenic microorganisms.

The theoretical basis for the application of fluorescent antibodies in medicine is established by applying three basically different sciences: immunology, chemistry, and optics.

Immunology must prove that the visible marking by means of fluorescent dyes does not change the specific affinity of the antibodies' molecules for their antigens.

Chemistry must solve first the question of the most appropriate dyes to be used for marking. Such dyes must have the following characteristics:

The fluorescence of the dye must be different from the self-fluorescence of the texture (usually bluish or white).

When the dye is combined with a molecule of a protein (antibody) it must not lose its fluorescence or change the specificity of the antibody.

The combination of the dye with the antibody must be constant.

These characteristics are found in fluorescin isotiocianate, which produces fluorescence in yellow-green color, and rhodamine B isocianate, which has red fluorescence.

The optics had to solve the question of observation of fluorescence under the microscope, for which it is necessary to have the following:

- A powerful source of light with many ultraviolet rays (200-1,000 watts).

- Filter which lets through ultraviolet rays and retains visible rays.

- Eyeglass filter, which protects the eye against ultraviolet rays.

- Condensor for a dark field.

- Ordinary monocular microscope.

When we observe under microscope preparations dyes with fluorescent antibodies, the antigen is illuminated in yellow-green color, and normal texture in blue, bluish, gray, and white color. In contrast coloring by means of rhodamine, the background (texture) is in red fluorescent color.

We used two methods for dyeing by means of fluorescent antibodies: direct and indirect method. We shall describe the direct method first.

Direct Method of Dyeing

When we apply the direct method, we have to have at our disposal as many marked specific antiserums as there are different bacterial, virus, fungus, protein, and other antigens which we examine. Figure 1 shows a diagram of the process of marking of antiserums.

The marked antibody reacts with the antigen, and this is reflected in the fluorescence in ultraviolet light. The "dyeing" takes place only when the marked antibody combines with the antigen, and therefore it is only an indication of that immunological phenomena. A marked anti-salmonella serum, when applied for example on a spread of mixed culture which also contains salmonella, reacts only to a homologous antigen. The combination of a marked salmonella antiserum to homologous salmonella is visible because of a specific fluorescence. Salmonella covered with marked antiserum shines (see Figure 2).

The direct method, which uses marked specific antiserum, is more simple, and it requires less procedures and control. It is the best method when applied to a large number of tests.

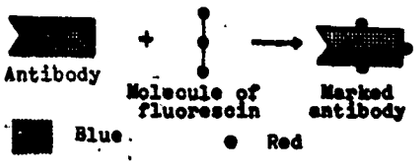


Figure 1

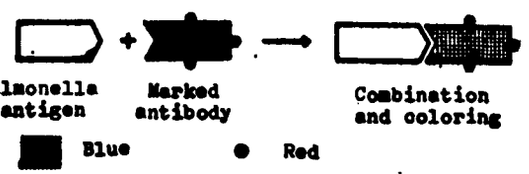


Figure 2

Indirect Method of Dyeing

In the indirect method, we do not mark a specific antiserum, for example anticolic serum applied to a rabbit. Instead, we mark an antiglobulin serum, which must be of the same origin as the antiserum. In our example, we must use a marked anti-rabbit globulin serum, because a specific anticolic serum is applied to a rabbit. The procedure in the indirect method includes two stages. During the first stage the germs (in our case escherichia coli) react to the antiserum. They become covered with a thin layer of rabbit globulin (invisible) and the germs treated in this way react again to the rabbit antiglobulin serum, which is manifested by the fact that the germs produce fluorescent light under ultraviolet rays. The course of the reaction is shown in a diagram in Figure 3.

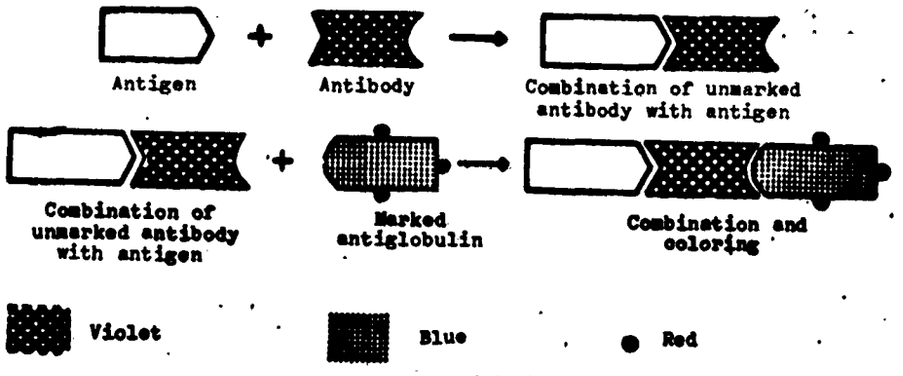


Figure 3

When we use the indirect method of dyeing by means of marked antibodies, we need as many marked antiglobulin serums as there are various species. When we test a human serum, we must use antihuman globulin serum. When we examine horse serum, we must use antihorse globulin serum, etc. As a routine procedure, it is enough to have three marked antiglobulins: anti-rabbit, anti-human, and anti-guinea pig. By using these, we can test generally all the serum and antigens in the area of human pathology.

Application in Bacteriology

The technique of dyeing by means of fluorescent antibodies has been applied broadly in bacteriology. We shall mention only a few examples. In cases of streptococcus infections, Moody and collaborators (14) found out that this method is entirely equivalent to the standard method of the determination of the group, with the difference that the results can be obtained 48 hours earlier. Whitaker and his collaborators (15) used this method to study an epidemic of child dysentery called E. coli 0127: B8. They found that the method is more sensitive. They needed much less time to determine the final diagnosis. Also they found that the germs can be discovered during an early stage of the disease. In recent times, Stulberg and collaborators (16) determined in epidemics of child dysentery four serological types E. coli in shavings of stool. These cases covered practically all cases of the epidemics.

This method is used to differentiate diphtheric and diphtheroid bacilli, gonococci, from other gram negative diplococci, to determine the type of pneumococci, to study staphylococci proteus, shigella, salmonella, pasteurella, brucella, listera, leptospira, malecmicosa and treponema palidium.

Application in Virology

This method was applied most profitably in virology to solve problems of etiology of primary atypical pneumonia. Although the most important agent of this clinical syndrome, the so-called Eatonov virus, was isolated more than 15 years ago, it has not been accepted as the cause of the disease because of certain laboratory difficulties. It wasn't until 1957 that Liu (17) determined definitely by using the method of dyeing by means of fluorescent antibodies that the eaton virus is the principal cause of the syndrome PAP. The same author used nasal-humous to diagnose influenza quickly (18). This method is applied in studies of the virus of parotiditis, especially its distribution in monkeys which have been infected experimentally. Coons and his collaborators (19) succeeded to identify R. Rickettsia in spreads of exudation or in frozen histological cuts.

Viruses which form compact bodies are very suitable for studies by means of fluorescent antibodies. Thus in many laboratories we study rabies by means of this technique. Small bodies and groups of rabies virus in marrow texture can be observed by this method faster and better than by the classical methods of dyeing.

This method is penetrating quite fast in all areas of virology. If there is no information about the value of this method for all viruses, it is primarily because it couldn't be done in such a short period of time.

Application in Parasitology

The method of dyeing by marked antibodies was applied profitably in parasitology as well. Goldman (20) succeeded to differentiate E. histolyticae and E. coli. Later on we succeeded to differentiate a large number of various types of amoebae.

Jackson and Lewert (21) report on a fast diagnosis of Trichinellae spirallis by means of marked rabbit antiserum.

Fife and Muschel (22) used the indirect method of dyeing by means of fluorescent antibodies to diagnose quickly tripanosomiasis (Trypanosoma cruzi). Laboratory diagnosis coincided with clinical diagnosis in thirty nine out of forty cases, while RVK was positive only 28 times. There was no cross reaction with normal and syphilitic serums.

Goldman (23) used this method to make a fast diagnosis of toxoplasmosis. He worked out a test of inhibited dyeing, which is based on the fact that an antibody combined with an antigen does not react any more to a fluorescent antibody. The test of inhibited dyeing by means of marked antibodies was more revealing than RVK.

Application in Mycology

Gordon studied the technique of dyeing by means of fluorescent antibodies in yeast (24) he found that there was antigenis affinity between Candidae albicans and Candidae tropicalis. The remaining Candidae (8 species) did not display any antigenis affinity. Fluorescent antiserum against C. albicans did not give any cross reaction with fifteen other genera.

Marshall and his collaborators (25) proved that Cryptococcus neoformans is homogenous in an antigen structure. Out of one hundred breeds, ninety nine reacted specifically by the method of dyeing through fluorescent antibodies. In the texture of deceased bodies, the germs could be discovered faster and more easily than by standard methods.

This method is applied also in studies of actynomyces bovis.

Technique of Dyeing

A. Direct Method

Material: a. Wet chamber,
b. Specific marked antiserum,
c. Specific unmarked antiserum.

Procedure:

1. Sample of antigen (texture culture, spread, crop, stool, suspension of texture, histological cuts) is attached to glass by acetone in cases of viruses, and by ethyl or methyl alcohol for other micro-organisms.

2. Pour the preparation with the mixer (marked specific antiserum), place it in a moist chamber for 30 minutes at 37° C.

3. Rinse the preparation three times with a physiological solution pH 7.2. Each rinsing takes 2 minutes.

4. Dry the preparation on glass.

5. Put a drop of glycerine on the preparation (9 parts of glycerine and one part of physiological solution) and cover with glass cover.

6. Observe under fluorescent microscope.

7. The positivity amounts to 4+, 3+, 2+, 1+, and 0. It represents the degree of fluorescence of the microorganism.

Control:

a) Pour unmarked specific antiserum over the preparation, place it in a moist chamber and let it incubate for 30 minutes at 37°C.

b) As an alternative control, we can use a combination for another antigen.

c) If the dyeing by means of a homologous combination is specific, there must be no fluorescence in the controls under a and b.

B. Indirect Method

Material: a. Moist chamber,
b. Specific antiserum (for example pneumococcus antiserum prepared for a rabbit).
c. Marked anti-rabbit globulin serum.

Procedure:

1. Apply a sample of antigen on glass.

2. Pour a few drops of pneumococcus serum over the preparation, place it in a moist chamber, and let it incubate for 30 minutes at 37°C.

3. Rinse the preparation three times with physiological solution pH 7.2.

4. Dry the preparation on glass.

5. Pour a few drops of anti-rabbit globulin serum over the preparation, place it in a moist chamber, and let it incubate for 30 minutes at 37°C.

6. Repeat the procedure sub points 3 and 4.

7. Place a drop of glycerine on the preparation and cover with a glass cover.

8. Observe under fluorescent microscope.

Control:

a) Instead of using a specific antiserum prepared from a rabbit, use a serum prepared from another species and proceed in the same way as indicated above.

b) Leave out operations under point 2 and 3 and continue the remaining operations.

c) The test is specific, if there is no fluorescence with control a and b.

The following table shows findings through direct and indirect methods.

Primary Treatment	Secondary Treatment			
	Marked rabbit antipneumococcus serum Type 2	Marked human pneumococcus serum Type 2	Marked anti-rabbit globulin serum	Marked anti-human globulin serum
Without treatment	Direct Test +	Direct Test +	Control -	Control -
Rabbit antipneumococcus serum Type 2	Control -	Control -	Indirect Test +	Control -
Human antipneumococcus Type 2	Control -	Control -	Control -	Indirect Test +

The direct and indirect methods described above are used to detect an unknown antigen by means of a known antiserum. The same method can be used also for titration of an unknown serum.

Legend:

a) Direct method: in the direct method there is no preliminary treatment by a specific antiserum. For that reason a direct test with a marked antipneumococcus serum of any origin (rabbit, human, horse, etc) must be positive, a test with a marked antihuman or anti-rabbit globulin serum must be negative, because such globulins do not contain specific antibodies against pneumococcus of Type 2.

b) Indirect method: in the indirect method the antigen (pneumococcus Type 2) is first treated with an unmarked rabbit or human antipneumococcus serum Type 2. Since the combination antigen-antibody took place during the first stage, the antigen cannot be dyed by subsequent treatment with marked rabbit or human antipneumococcus serum. There is specific coloring of the antigen in the indirect method only when we use marked antiglobulin serum in the second stage, namely rabbit marked antiglobulin serum, if the primary treatment involves rabbit antipneumococcus serum Type 2, and human antiglobulin serum, if the primary treatment involves human antipneumococcus serum Type 2. There must be no coloring, if instead of a marked anti-human globulin serum we use a marked anti-rabbit globulin serum (second example). If the marked antiglobulin serum does not correspond to the serum which is used in the primary treatment (rabbit antiserum - marked human antiglobulin or human antiserum - marked rabbit antiglobulin), the procedure is used to control the specificity of coloring of fluorescent antibodies.

Work received 28 February 1961.

Literature

1. Marrack, J.: Nature, 133, 292, 1934.

2. Coons, A. H., H. J. Creech, R. N. Jones: Proc. Soc. Exp. Biol. Med., 47, 200, 1941.

3. Creech, H. J., R. N. Jones: J. Amer. Chem. Soc., 62, 1970, 1940.

4. Coons, A. H., H. J. Creech, R. N. Jones, E. E. Berliner. J. Immunol., 45, 159, 1942.

5. Coons, A. H., M. H. Kaplan: J. Exp. Med., 91, 15, 1950.

6. Kaplan, M. H., A. H. Coons, H. W. Deane: J. Exp. Med., 91, 15, 1950.

7. Riggs, J. L., Synthesis of fluorescent compounds and their use for labelling antibody. Masters Thesis, Univ. of Kansas, 1957.

8. Marshall, J. D. Jr., W. C. Eveland, C. W. Smith: Proc. Soc. Exp. Biol. Med., 98, 898, 1959.

9. Silverstein, J. O.: J. Histochem., 5, 94, 1957.

10. Hiramato, R., K. Engel, D. Pressman: Proc. Soc. Ehp. Biol. Med., 97, 611, 1958.

11. Chadwick, C. S., M. G. McEntegart, R. C. Nairn: Lancet, No. 7017, 412, 1958.

12. Smith, C. W., J. D. Jr. Marshall W. C. Eveland: Proc. Soc. Exp. Biol. Med., 98, 898, 1958.

13. Marshall, J. D. Jr.: J. Exp. Med., 94, 21, 1951.

14. Moody, M. D., E. C. Ellis, E. L. Uddyke: Bacter. Proc. s. 135, 1958.

15. Whitaker, J. R. H. Page, C. S. W. W. Zuelzer: J. Dis. Child., 95, 1, 1958.

16. Stulberg, C. W., F. Cohen, R. H. Page: Bact. Proc.

17. Liu, C.: J. Exp. Med., 106, 455, 1957.

18. Liu, C.: Proc. Soc. Exp. Biol. Med., 92, 683, 1956.

19. Coons, A. H., J. C. Snyder, F. S. Cheever, E. S. Murray: J. Exp. Med., 91, 31, 1950.

20. Goldman, M.: Amer. J. Hyg., 59, 318, 1954.

21. Jackson, G. J., R. M. Lewert: J. Parasit., 43:5, sec. 2, 43, 1957.

22. Fife, E. H. Jr., L. H. Muschel: Proc. Soc. Exp. Biol. Med., 101, 540, 1959.

23. Goldman, M.: J. Exp. Med., 105, 557, 1957.

24. Gordon, M. A.: Proc. Soc. Exp. Biol. Med., 97, 694, 1958.

25. Marshall, J. D. Jr., L. Iverson, W. C. Eveland, A. Kase: Amer. J. Path., 35, 684, 1959.

- END -